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Abstract

Cocaine addiction is a chronically relapsing disorder that is associated with harmful consequences. Relapses occur frequently and effective pharmacotherapies are currently sparse. Preclinical studies suggest that altered glutamatergic signaling is crucial for the maintenance of cocaine self-administration. However, the translational validity of these models is currently unknown. Therefore, we investigated potential differences of glutamate, glutamine, and further metabolite levels in the pregenual anterior cingulate cortex (pgACC) and the right dorsolateral prefrontal cortex (rDLPFC) of chronic cocaine users and controls using the ProFit 2.0 tool in combination with 2D J-resolved single-voxel ^{1}H-magnetic resonance spectroscopy at 3T and voxel tissue composition and relaxation correction. Glutamate and glutamine levels did not differ between cocaine users and controls but higher weekly cocaine use and higher cocaine hair concentrations were associated with lower glutamine/creatine ratios in the pgACC. Interestingly, cocaine users exhibited higher glucose/total creatine ratios than controls in the pgACC and higher choline/creatine ratios in the pgACC and rDLPFC. These results imply that cocaine use is associated with altered cortical glucose metabolism and membrane turnover. Finally, cocaine use over the past six months appears to decrease cortical glutamine levels indicating changes in glutamate cycling.

Keywords: anterior cingulate cortex, choline, cocaine addiction, glutamate, glutamine, magnetic resonance spectroscopy

Introduction

A hallmark of cocaine addiction is the compulsive seeking and taking of cocaine despite the occurrence of harmful consequences. Preventing the frequently occurring relapses remains one of the most challenging issues in treating cocaine addiction and few effective pharmacotherapies are currently available (Hyman & Malenka 2001). The persisting vulnerability to relapse presumably arises from conditioning processes where neutral environmental stimuli become associated with cocaine-related stimuli and eventually increase the risk for relapse by inducing strong craving urges for the drug (Goldstein & Volkow 2011; Volkow *et al.* 2006). Seminal findings from preclinical studies have indicated that alterations in glutamatergic signaling may be of particular importance for relapse vulnerability (Gipson *et al.* 2013; Kalivas 2009). In particular, the glutamatergic projection from the prefrontal cortex (PFC) to the nucleus accumbens (NAc) core appears to hold a crucial role in mediating drug-seeking in rodents (Kalivas 2009; Sun & Rebec 2006). Accordingly, after extinction from repeated cocaine self-administration, the basal burst activity of PFC neurons (Sun & Rebec 2006) and extracellular glutamate levels in the NAc core were reduced (Baker *et al.* 2003), and the ability to induce synaptic plasticity in NAc medium spiny neurons was markedly impaired in rats (Gipson *et al.* 2013). Contrarily, in response to cocaine-conditioned cues a large increase of synaptic glutamate release from prefrontal afferents to the NAc core (McFarland *et al.* 2003) and rapid, transient long-term potentiation (LTP) have been observed, the latter of which was associated with the intensity of reinstated cocaine-seeking (Gipson *et al.* 2013).

These preclinical findings may parallel findings from neuroimaging studies in humans where chronic cocaine users (CU) exhibited reduced glucose metabolism in the PFC during protracted abstinence (Volkow *et al.* 1992). In contrast, PFC activity and dopaminergic changes were strongly enhanced upon exposure to drug-associated cues, which was associated with the strength of drug craving (Volkow *et al.* 2006). It has recently been posited that the loss of synaptic plasticity in response to nondrug-associated stimuli may be responsible for the difficulty of CU to exert alternative and more appropriate behaviors competing with drug relapse, while the LTP induced by drug-associated stimuli may be linked to the overwhelming desire to procure the drug (Gipson *et al.* 2013). However, to what extent the glutamatergic abnormalities observed during withdrawal and cue-induced

drug-reinstatement in rats also occur in human CU is largely unknown. In addition to metabolic abnormalities, also structural alterations have been observed in chronic CU including reduced white and gray matter volumes particularly in the PFC (Lyoo *et al.* 2004; Makris *et al.* 2008). Thus, identifying and understanding glutamatergic and other neurometabolic alterations associated with the vulnerability to relapse may contribute to the development of novel medications for the treatment of cocaine addiction.

Proton magnetic resonance spectroscopy (^{1}H-MRS) is a suitable technique to non-invasively quantify neurotransmitters and neurometabolites in vivo in humans. A number of ^{1}H-MRS studies have investigated the combined signal of glutamate and glutamine (Glx), the most abundant excitatory neurotransmitter and its metabolic product, or solely glutamate as well as other neurometabolite levels in human CU (Chang *et al.* 1999; Martinez *et al.* 2013; Schmaal *et al.* 2012; Yang *et al.* 2009). Regarding glutamatergic abnormalities, prior studies reported both decreased (Yang *et al.* 2009) and increased (Schmaal *et al.* 2012) glutamate levels in the rostral vs. dorsal anterior cingulate cortex (ACC) of CU expressed as glutamate/total creatine (tCr) ratios. Moreover, a recent study quantified Glx in the striatum of 15 dependent crack CU, but failed to find significant differences between CU and controls (Martinez *et al.* 2013). Interestingly, a study with non-human primates was able to separately resolve glutamate and glutamine in the putamen (Liu *et al.* 2011). Squirrel monkeys who had been exposed to cocaine for nine months exhibited reduced glutamate/tCr levels in the putamen after one month, but had increased glutamate/tCr levels after six and nine months and increased glutamine/tCr levels after nine months compared to baseline (Liu *et al.* 2011). Extracellular glutamate is normally taken up and converted into glutamine by astrocytes and is subsequently released to be taken up and reconverted to glutamate by neurons. Although it is not possible with ^{1}H-MRS to distinguish the metabolic from the neurotransmitter pool, separate quantification of glutamate and glutamine may provide additional information regarding altered neuroenergetics and glutamate-glutamine cycling. Further metabolites of interest include γ-aminobutyric acid (GABA), *N*-acetylaspartate (NAA), total choline (tCho), and myo-inositol. GABA, the primary inhibitory neurotransmitter, and NAA, a putative marker for neuronal function or loss, were decreased in the frontal cortex of CU (Ke *et al.* 2004; Simmons *et al.* 1991). tCho, implicated in membrane turnover,

and myo-inositol, involved in phosphatidylinositol second messenger signaling and osmoregulation, have been shown to be increased in CU (Ross 1991). Lower NAA and higher tCho levels could reflect the above-mentioned gray and white matter reductions in CU (Lyoo *et al.* 2004; Makris *et al.* 2008).

To reliably detect the main metabolites of interest including glutamate, glutamine, GABA, NAA, tCho, and myo-inositol in the pregenual anterior cingulate cortex (pgACC) and the right dorsolateral PFC (rDLPFC) in chronic CU and healthy controls (HC), we applied an enhanced version of PRior knOwledge FITting tool (ProFit) in combination with two-dimensional (2D) J-resolved single-voxel ^{1}H-MRS (Fuchs *et al.* 2013). For these metabolites, specific hypotheses could be generated based on prior studies. We further investigated ten additional exploratory metabolites that have not been systematically investigated thus far: *N*-acetylaspartylglutamate (NAAG), glucose, lactate, scyllo-inositol, taurine, glycine, glutathione, phosphoethanolamine, aspartate, and ascorbic acid. The pgACC and the rDLPFC were selected as regions-of-interest because of their key involvement in cocaine dependence: Chronic CU have been shown to exhibit reduced glucose metabolism in the pgACC and DLPFC during sustained withdrawal (Volkow *et al.* 1992), whereas upon the presentation of drug-associated cues increased functional activity of the pgACC has been observed (Volkow *et al.* 2006). The pgACC holds an important role in emotion regulation and is assumed to mediate stress reactivity, attentional bias towards drug-associated stimuli, and disrupted ability to update the reward value of non-drug reinforcers in addicted drug users (Goldstein & Volkow 2011). The DLPFC is implicated in top-down control and may be associated with impulsive and compulsive behaviors in cocaine-addicted individuals, the processing of negative affect including cocaine craving, and enhanced motivation to obtain cocaine opposed to other reinforcers and drug-related learning (Goldstein & Volkow 2011).

Based on prior results, we hypothesized that: 1) CU exhibit lower glutamate, glutamine, GABA, and NAA concentration ratios and increased tCho and myo-inositol concentration ratios, 2) lower glutamate and glutamine concentration ratios are associated with craving scores, duration and quantity of cocaine use. A secondary goal was to investigate the influence of potential co-factors on metabolite concentration ratios including alcohol and tobacco, and post-acute cocaine and cannabis effects.

Materials and methods

Subjects

The study sample comprised 18 male drug-naïve HC and 18 male chronic CU who participated in the longitudinal Zurich Cocaine Cognition Study ($ZuCo^2St$)(Hulka *et al.* 2014a; Preller *et al.* 2013; Vonmoos *et al.* 2013) including a positron emission tomography (PET) study (Hulka *et al.* 2014b). Inclusion criteria for CU were 1) age 20-50 years, 2) DSM-IV diagnoses of cocaine abuse/dependence (Wittchen *et al.* 1997), 3) no polytoxic drug use and medication affecting the CNS, 4) no current or previous Axis I DSM-IV psychiatric disorder (other than cocaine abuse/dependence, alcohol abuse, nicotine dependence, history of depression), 5) no family history of a severe psychiatric disorder such as schizophrenia, bipolar or obsessive-compulsive disorder, 6) no neurological disorder or head injury, and 7) no magnetic resonance imaging (MRI) contraindication. The same inclusion criteria applied to the HC except for criterion 2). In addition, HC were excluded if they regularly engaged in illegal drug use with exception of cannabis use. Participants were instructed to abstain from illegal drugs for >3 days and >24 hours from alcohol. Urine samples were collected to control for recent drug use. To objectively characterize drug use over the past six months, hair samples (6cm) were collected and analyzed with liquid chromatography-mass spectrometry (for details Vonmoos *et al.* 2013). The study was approved by the Ethics Committee of the Canton of Zurich and was carried out in accordance with the Declaration of Helsinki. All participants provided written informed-consent before inclusion and received financial compensation.

Clinical assessment

Trained psychologists screened all participants for the presence of Axis-I psychiatric disorders using the *Structured Clinical Interview for DSM-IV Disorders* (Wittchen *et al.* 1997). Drug use was assessed with the *Interview for Psychotropic Drug Consumption* (for details Vonmoos *et al.* 2013). The *Beck Depression Inventory* (BDI) was used to assess symptoms of depression (Beck *et al.* 1961). The *Symptom Checklist-90-R* (SCL-90-R) served as a screening measure of general psychiatric symptoms (Franke 1995). The brief version of the *Cocaine Craving Questionnaire* (CCQ) was applied

to measure current cocaine craving (Sussner *et al.* 2006). The *Fagerström Test for Nicotine Dependence* (FTND) was used to determine severity of nicotine dependence (Heatherton *et al.* 1991).

Image acquisition and data analyses

MRI Acquisition: For each participant, a T1-weighted three-dimensional fast gradient echo MRI scan (180 slices, FOV=220mm, matrix=224x224 reconstructed to 256x256, voxel size=0.98x0.98x1.5mm) was acquired on a Philips Achieva 3T whole-body scanner equipped with a transmit/receive head coil (Philips Healthcare, Best, The Netherlands) to rule out structural abnormalities and to place the ^{1}H-MRS voxels of interest. Prior to the MRI and ^{1}H-MRS acquisition, all participants underwent a PET scan (Hulka *et al.* 2014b).

^{1}H-MRS Acquisition and Processing: Single voxel ^{1}H-MRS data were acquired for the predefined volume-of-interest (VOI) of 25x18x20mm^{3}=9.0ml in the bilateral pgACC and the rDLPFC (**Figure 1A and B**) using a birdcage transmit-receive head coil with a maximum B_1=20μT. In order to quantify the main metabolites of interest glutamate, glutamine, GABA, NAA, tCho, and myo-inositol as well as ten exploratory metabolites including NAAG, glucose, lactate, scyllo-inositol, taurine, glycine, glutathione, phosphoethanolamine, aspartate, and ascorbid acid, a maximum echo-sampled 2D J-resolved point-resolved spectroscopy (JPRESS) sequence (TR of 1600 ms, TE ranging from 26 to 224 ms with step size of 2 ms, 100 encoding steps, 8 averages per step) with VAPOR water and interleaved inner volume suppression was applied. The improved quantification approach based on ProFit 2.0 has been validated elaborately and methodological details are described elsewhere (Fuchs *et al.* 2013). The ^{1}H-MRS acquisitions lasted 22 minutes for each brain region. Prototypical projections of 2D J-PRESS spectra including spectral fit and residue of the pgACC and rDLPFC are depicted in **Figure 1A and B** and a 2D JPRESS spectrum of the pgACC including fit and residue in **Figure 2**.

Metabolite levels were referenced to tCr (as reported in the main manuscript) and to internal water (**supplementary information**, **SI**), yielding comparable results, and a segmentation based volume tissue composition and relaxation correction was applied for both reference standards creatine and water (Gasparovic *et al.* 2006). Prior ^{1}H-MRS studies did not find significant tCr concentration differences between CU and HC (Meyerhoff *et al.* 1999; Schmaal *et al.* 2012; Yang *et al.* 2009). In

addition, in our study, the tCr levels in CU and HC did not differ when referenced to internal water even after correction for age, smoking status, and gray and white matter volumes (p>.42, see **Table 3**). The following equations, adopted from Gasparovic et al. (2006), were applied for the use of H_2O (equation 1) and tCr (equation 2) as internal concentration references, with S_{M_obs} standing for observed metabolite signal, f for fraction including volume share and tissue type specific concentration differences for water (equations 5-7 in Gasparovic *et al.* 2006) and creatine (equation 3) references, GM for gray matter, WM for white matter, CSF for cerebrospinal fluid, and R for relaxation attenuation factor including T1 and T2 relaxation correction for water and creatine:

(1) $[M_{\text{referenced to } H2O}]=(S_{M\_obs}\times(f_{GM\_H2O}\times R_{H2O\_GM}+f_{WM\_H2O}\times R_{H2O\_WM}+f_{CSF\_H2O}\times R_{H2O\_CSF})\times 2x55.51[\text{mol/kg}])/(S_{H2O\_obs}\times(1-f_{CSF}))$

(2) $[M_{\text{referenced to tCr}}]=S_{M\_obs}\times(f_{GM\_Cr}\times R_{Cr\_GM}+f_{WM\_Cr}\times R_{Cr\_WM})\times 3\times 7.62[\text{mol/kg}]/S_{tCr\_obs}$

(3) $f_{GM\_Cr}=0.00095*f_{GM\_Vol\_Cr}\times 1/(f_{GM\_Vol\_Cr}\times 0.00095+f_{WM\_Vol\_Cr}\times 0.00051)$

$f_{WM\_Cr}=0.00051*f_{WM\_Vol\_Cr}\times 1/(f_{GM\_Vol\_Cr}\times 0.00095+f_{WM\_Vol\_Cr}\times 0.00051)$

The resulting metabolite concentrations were reported in arbitrary units since only a tissue type non-specific but individual T2 relaxation correction has been performed by ProFit 2.0 for all reported metabolites (Fuchs *et al.* 2013), but no T1 relaxation correction or correction for the number of contributing protons was applied for the metabolites (only for the reference standards) as for most of them T1 relaxation times are still unknown.

The Cramér-Rao lower bounds (CRLBs) served as the quality criterion to determine the reliability of the fit for each metabolite peak. Metabolite estimates with CRLBs >20% were excluded. Due to movement artifacts or CRLBs >20%, one spectrum of a control subject from the pgACC and five spectra of CU from the rDLPFC had to be excluded from the statistical analyses.

Statistical analysis

Statistical analyses were carried out with the PASW 19.0 software (SPSS Inc.). Demographic, clinical, and drug use data of the two groups were analyzed with independent T-tests and frequency analyses (Pearson's Chi^2-test). Analyses of covariance (ANCOVA) with age, smoking status, gray and white matter volumes as covariates were conducted to compare ^{1}H-MRS metabolite levels between the two groups. For the main metabolites of interest one-tailed tests were carried out and for the exploratory metabolites two-tailed tests.

For the main metabolites of interest, specific hypotheses regarding the direction of the effect were derived based on prior data, wherefore one-tailed tests were carried out. For the exploratory metabolites two-tailed tests conducted. Potential associations of drug use patterns and clinical measures with ^{1}H-MRS metabolite levels were examined with partial correlation analyses (adjusted for age). The *p*-value was set at .05. Drug use parameters (weekly use, last use, cumulative doses) were log-transformed as the assumptions of parametric distribution and homoscedasticity were not met and the constant 1 was added because the data contained 0 values. Effect sizes (Cohen's *d*) were calculated with G*Power 3.1 (Faul *et al.* 2007).

Results

Subject characteristics and drug use patterns

HC and CU did not differ with regard to age, body mass index, years of education, BDI and SCL-90 scores, smoking status, and smoking severity **(Table 1)**. Nine (50%) CU met the DSM-IV criteria for current cocaine dependence and nine (50%) CU met the criteria for cocaine abuse. All except one CU who smoked cocaine reported to administer the drug nasally. Legal and illegal drug use patterns are shown in **Table 1 and 2**. CU did not differ from HC with regard to tobacco consumption. Contrarily, CU reported higher weekly alcohol consumption [$t(34)=-2.36$, $p<.05$, $d=.79$] and higher cumulative doses of cannabis [$t(34)=-2.18$, $p<.05$, $d=.73$]. Hair toxicology analyses capturing the past six months confirmed that cocaine was the primary drug of choice. Methamphetamine and opiates were not detected at all, amphetamine levels were below the threshold of reliable detection >200pg/mg (n=3), and MDMA levels were small to moderate (n=6). Notably, urine toxicology analyses revealed that 44% of the CU tested positive for recent cocaine use, and 13% of the HC and 33% of the CU tested positive for cannabis use.

Tissue composition and regional concentration ratios of the main metabolites

Groups neither differed with regard to gray matter, white matter, and cerebrospinal fluid volumes in the pgACC ($p>.35$) nor the rDLPFC ($p>.14$)(**Table 3**). Concentration ratios for the main metabolites of interest of HC and CU are shown in **Table 3** (concentration ratios of all metabolites referenced to H_2O are shown in **Table S2).** An ANCOVA with age, smoking status, gray matter volume, and white matter volume as covariates revealed that in the pgACC, CU showed a higher tCho/tCr ratio ($d=.54$) in comparison to HC. Also in the rDLPFC, CU exhibited a higher tCho/tCr ratio ($d=.72$; **Figure 3B**) and, additionally, by trend a higher myo-inositol/tCr ratio ($d=.60$) than HC. No significant group differences were observed for glutamate or glutamine levels (**Figure 4A-D**).

Correlations between drug use patterns and main metabolites

Only subjects who used the drug of interest were included in the analyses in order to avoid spurious correlations. *Cocaine use:* In the pgACC voxel of CU, higher quantity of weekly cocaine use

(*r*=-.59, *p*<.05, *n*=17; **Figure 5A**) and total cocaine hair concentration (*r*=-.57, *p*<.05, *n*=17; **Figure 5B**) were associated with lower glutamine/tCr ratios. In the rDLPFC voxel, higher weekly cocaine use (*r*=-.67, *p*<.05, *n*=13; **Figure 5C**) and total cocaine hair concentration (*r*=-.64, *p*<.05, *n*=13; **Figure 5D**) were associated with lower myo-inositol/tCr ratios. *Alcohol use:* In the pgACC, longer alcohol abstinence duration was associated with higher GABA/tCr ratios (*r*=.61, *p*<.01, *n*=19). In the rDLPFC, longer alcohol abstinence duration was associated with lower tCho/tCr ratios (*r*=-.37, *p*<.05, *n*=31). No significant correlations were found between brain metabolite concentrations and tobacco or cannabis use parameters.

Correlations between demographic/clinical measures and main metabolites

In the pgACC voxel, older age was associated with a lower glutamine/tCr ratio (*r*=-.42, *p*<.05, *n*=34) and higher scores of the global severity index in the SCL-90 were associated with higher tCho/tCr ratios (*r*=.37, *p*<.05, *n*=35). In the rDLPFC voxel, more general psychiatric symptoms (SCL-90 scores) were associated with a higher NAA/tCr ratio (*r*=.40, *p*<.05, *n*=32).

Regional concentration ratios of the exploratory metabolites

Concentration ratios of the ten exploratory metabolites are presented in **Table S1.** In the pgACC, CU displayed a higher glucose/tCr ratio (*d*=.76; **Figure 3A**) than HC. In the rDLPFC, CU and HC did not differ with regard to any metabolite concentration ratios.

Correlations between drug use patterns and exploratory metabolites

Alcohol use: In the pgACC, longer alcohol abstinence duration was associated with a higher NAAG/tCr ratio (*r*=.37, *p*<.05, *n*=31). In the rDLPFC, higher weekly alcohol use was associated with a higher lactate/tCr ratio (*r*=.44, *p*<.05, *n*=29) and a higher phosphoethanolamine/tCr ratio (*r*=.42, *p*<.05, *n*=26). None of the cocaine, cannabis or tobacco use variables were correlated with exploratory metabolite concentration ratios.

Potential Co-Factors

Recent cocaine use: An additional ANCOVA with age, smoking status, gray matter volume, and white matter volume as covariates was carried out, comparing metabolite concentration ratios between HC (n=17), CU with a negative cocaine urine screen (n=10), and CU with a positive cocaine urine screen (n=8). In the pgACC, there were no significant group differences for any of the main or exploratory metabolite concentration ratios indicating that recent cocaine use did not measurably alter metabolite levels in CU ($p>.05$). In the rDLPFC, CU who tested positive for cocaine (mean:4893.69, SEM:±227.48, n=6; $F(2/22)=6.35$, $p<.01$) had increased tCho/tCr ratios compared to HC (3985.60±138.25, n=16, $p<.05$, $d=1.30$) and CU who tested negative for cocaine (3945.92 ±211.18, n=7, $p<.05$, $d=1.34$).

Recent cannabis use: In both the pgACC and rDLPFC, main and exploratory metabolite levels of CU with a negative and positive urine screen for cannabis did not significantly differ from HC with a negative urine screen for cannabis ($p>.05$).

Smoking status: Smokers and nonsmokers did not significantly differ with regard to any of the main and exploratory metabolites in the pgACC and rDLPFC ($p>.05$).

Discussion

In the present study, we investigated potential glutamatergic and other neurometabolic alterations associated with chronic cocaine use, by using 2D JPRESS ^{1}H-MRS combined with an improved quantification approach based on ProFit 2.0 and a comprehensive voxel tissue composition and relaxation correction approach enabling a separate quantification of glutamate and glutamine. Contrary to our expectation, glutamate and glutamine concentration ratios in the pgACC and rDLPFC did not significantly differ between CU and HC. However, higher weekly cocaine use and higher total concentrations of cocaine and its metabolites in the hair samples were significantly associated with lower glutamine concentration ratios in the pgACC indicating glutaminergic alterations in more severe CU. Moreover, CU exhibited significantly higher tCho concentration ratios than HC in the pgACC and rDLPFC and higher glucose/tCr ratios in the pACC. Finally, in the rDLPFC, CU displayed a trend for higher myo-inositol concentration ratios compared to HC, while in CU myo-inositol levels were negatively correlated with weekly cocaine use and cocaine concentration in the hair.

Our non-significant group differences regarding glutamate and glutamine concentration ratios differ from two inconsistent prior studies with human CU: Yang et al. (2009) investigated a similar VOI in the bilateral pgACC of 14 chronic crack cocaine users and found a significant glutamate reduction (15.9%) in CU compared to HC. In contrast, Schmaal et al. (2012) reported increased glutamate concentrations in a more dorsal part of the left ACC. One explanation for the non-significant group difference in the present study could be low statistical power. Alternatively, the varying results may also be due to different drug use patterns, abstinence durations, addiction severity, the selection of different portions of the ACC (dorsal vs. rostral), and methodological differences including the selection of different covariates and ^{1}H-MRS acquisition and processing routines as well as the lack of tissue composition corrections in prior studies. For instance, the CU who participated in the studies of Yang et al. (2009) and Schmaal et al. (2012) all met the criteria for cocaine dependence, used substantially higher monthly doses of cocaine and other illegal drugs, and smoked mostly crack cocaine, whereas only 9 out of 18 CU of the present study met the dependence criteria, predominantly

administered cocaine nasally and in lower quantities, and had little co-use of other drugs. Therefore, it is possible that the less pronounced (poly)drug use in the present CU sample did not lead to measurable glutamate alterations. However, it is noteworthy that a recent study with 15 dependent CU also did not find significant differences of Glx concentrations between CU and HC in the striatum (Martinez *et al.* 2013). Also in contrast to the findings of Yang et al. (2009) where cocaine use duration correlated positively with glutamate levels, we did not find a significant correlation between cocaine use duration and glutamate or glutamine. Moreover, whereas Schmaal et al. (2012) found a negative correlation between self-reported cocaine use over the past six months and glutamate, we observed a negative association of self-reported weekly use of cocaine and cocaine concentrations in the hair samples (representing the use during the past six months) with glutamine but not glutamate in the pgACC. A possible explanation could be that less glutamate is taken up by glial cells and converted into glutamine due to down-regulated glutamate transporters as has been shown in rats after chronic cocaine administration (Knackstedt *et al.* 2010). The correlation found in our study could indicate that the down-regulation of glutamate transporters may be particularly pronounced in CU with more frequent and stronger cocaine use. However, this conjecture has to be tested in controlled animal studies. The fact that weekly cocaine use and cocaine concentrations in the hair samples but not cumulative doses or the duration of cocaine use correlated negatively with glutamine/tCr concentration ratios in the pgACC could suggest that recent cocaine use may be more tightly linked to glutamate-glutamine cycling than cumulative doses and duration of use. It is noteworthy that increased glutamate levels have been detected in the dorsal ACC of alcohol- and opiate-dependent patients specifically during withdrawal (Hermann *et al.* 2012a; Hermann *et al.* 2012b). These findings indicate that the stage of addiction may be relevant to detect changes in Glu signals.

The present study yielded higher tCho concentration ratios in the pgACC and rDLPFC of CU in comparison to HC, and a trend for increased myo-inositol in the rDLPFC of CU (p=.06). Our findings corroborate two prior studies reporting that dependent CU exhibited higher tCho levels in the PFC than HC (Chang *et al.* 1999; Meyerhoff *et al.* 1999). In contrast, Yang et al. (2009) did not observe these differences in their study. tCho is a composite measure of glycerophosphocholine,

phosphocholine, a small amount of free choline, and a negligible contribution from acetylcholine (Ross 1991). tCho is involved in membrane turnover and has been shown to be elevated in strokes, neoplasms, demyelination, inflammation, and gliosis (Gill *et al.* 1989). Myo-inositol is a pentose sugar putatively involved in osmoregulation, glucose storage (Ross 1991), and the calcium-mobilizing phosphatidylinositol second-messenger system (Berridge & Irvine 1989). Elevations in myo-inositol levels have been reported for low-grade brain tumors, demyelinating diseases, and Alzheimer's disease and have been hypothesized to reflect glial hypertrophy or proliferation as glial cells express higher myo-inositol levels than neurons. Due to the vasoconstrictive effects of cocaine, CU have an increased risk for cerebrovascular complications (Martin-Schild *et al.* 2010). Potentially, elevated tCho and myo-inositol levels of CU may be explained by undetected hemorrhagic or ischemic micro strokes and hence subsequent demyelination and gliosis.

The increased tCho and myo-inositol levels may also be in line with previous studies reporting marked structural alterations in chronic CU including lower white and gray matter densities in fronto-limbic brain areas (Lyoo *et al.* 2004; Makris *et al.* 2008). Interestingly, the elevated tCho concentrations found in the present study were more pronounced in the rDLPFC than the pgACC. It is noteworthy that in a prior study, the cortical thickness of 20 dependent CU was particularly reduced in the rDLPFC, which also correlated with abnormal cognitive control (Makris *et al.* 2008).

With regard to NAA, CU and HC of the present study did not significantly differ, which is in accordance with the findings of Yang et al. (2009) and Schmaal et al. (2012) but not with two older studies reporting decreased NAA levels in the PFC (Chang *et al.* 1999) and DLPFC of CU (Meyerhoff *et al.* 1999). NAA, one of the most abundant peptides, is primarily found in neurons, axons, and dendrites and is a putative marker for neuronal integrity (Simmons *et al.* 1991). With regard to GABA, CU and HC did not significantly differ in our study, which is in contrast to a prior study reporting reduced GABA levels in CU (Ke *et al.* 2004). Altogether, the fact that CU displayed increased tCho and myo-inositol levels particularly in the rDLPFC but no significant changes in NAA may indicate that astroglial signaling alterations reflected by cocaine-associated glutamine ratio reductions may be more prominent than perturbation of neuronal function or neuronal damage.

The investigated exploratory metabolites did not differ between CU and HC with exception of glucose. In the pgACC, glucose/tCr ratios were higher in CU compared to HC, which possibly indicating lower metabolic activity or glucose consumption rate. This notion would be in line with PET studies reporting reduced glucose metabolism in the PFC of dependent CU during protracted withdrawal (Volkow *et al.* 1992). However, changes of glucose metabolism in the ACC of CU measured with ^{1}H-MRS have not been reported so far warranting further investigation.

A secondary aim of the present study was to investigate the influence of potential co-factors on metabolite levels including the use of other legal and illegal substances and urine toxicology results. Our exploratory results revealed that longer alcohol abstinence was associated with higher GABA and NAAG concentration ratios in the pgACC, as well as lower tCho concentration ratios in the rDLPFC, and higher weekly alcohol use was associated with higher lactate and phosphoethanolamine levels in the rDLPFC. The preliminary findings of the present study may be in line with studies showing that initially reduced GABA and NAA levels normalized with longer alcohol abstinence (Bendszus *et al.* 2001). The association between longer alcohol abstinence duration and lower tCho levels observed in our study is in accordance with prior findings showing that higher alcohol consumption in non-abstinent alcohol users and in light-drinking controls was associated with higher frontal Cho levels (Ende *et al.* 2006), whereas recently detoxified and 1-month-abstinent alcohol dependent individuals exhibited reduced or unchanged Cho levels (Bendszus *et al.* 2001; Schweinsburg *et al.* 2000). The association of higher weekly alcohol use with higher lactate and phosphoethanolamine levels in the rDLPFC could reflect oxidative stress, hypoxia or ischemia.

Whereas recent cocaine use did not appear to influence metabolite levels in the pgACC, in the rDLPFC tCho was particularly elevated in CU who had tested positive for recent cocaine use. Hence, we cannot fully rule out that the higher tCho/tCr concentrations at least in the rDLPFC of CU are partially explained by post-acute cocaine effects. Though, the elevated tCho levels in the pgACC seem to be unaffected by recent cocaine use.

With regard to the clinical variables and their association with metabolite levels, we found that cocaine craving scores did not correlate with glutamate and glutamine levels. It is noteworthy that

craving scores were not very high in the present CU sample as we did not actively trigger craving. Additionally, a substantial part of the CU tested positive for recent cocaine use. Interestingly, we found that more pronounced general psychiatric symptoms were associated with higher tCho levels in the pgACC and higher NAA levels in the rDLPFC. These results were mainly driven by the CU indicating that disrupted membrane turnover and neuronal NAA metabolism may be linked to the development of psychiatric symptoms.

A number of limitations of the present study need to be considered. First, the sample size of 36 participants is modest, yet comparable to other ^{1}H-MRS addiction studies. Second, the quantification of up to 9 of the GABA and glucose datasets exceeded CRLBs of 20% and had to be excluded from further analyses, decreasing the sample size and interpretability for these metabolites. Third, our CU sample may not have been suitable to detect glutamatergic alterations potentially induced by severe cocaine consumption, because only half of the CU met the criteria for cocaine dependence and overall, the CU of the present study used substantially less cocaine and other illegal drugs in comparison to CU examined in prior studies. Fourth, although we conducted additional analyses to investigate if co-factors such as recent consumption of cocaine and cannabis have an effect on metabolite concentrations, we cannot fully exclude the possibility that recent drug use may have masked group differences. Finally, although we excluded individuals meeting the DSM-IV criteria for alcohol dependence and CU had relatively low cocaine craving scores, we cannot completely rule out that alcohol and potentially cocaine withdrawal symptoms may have upregulated prefrontal glutamate input, possibly accounting for the non-significant group difference and contradictory findings across studies.

In conclusion, glutamate and glutamine concentration ratios did not significantly differ between CU and HC but higher weekly cocaine use and higher cocaine hair concentrations were associated with lower glutamine concentration ratios in the pgACC. Moreover, chronic CU exhibited significantly higher glucose concentration ratios in the pgACC and higher tCho concentration ratios in the pgACC and rDLPFC than HC. Altogether, the findings of the present study suggest that higher

quantity and frequency of cocaine used over the past six months appears to linearly decrease glutamine concentrations and that cocaine use might be associated with altered glucose metabolism and perturbations in membrane turnover. Future investigations should attempt to obtain a more complete understanding of how neurometabolic concentrations may differ between different stages of cocaine addiction. Moreover, ^{13}C-MRS could provide valuable information with regard to compartmental glutamate-glutamine changes in neurotransmitter and metabolic pools. Lastly, applying ^{1}H-MRS in the NAc of human CU would be of particular interest, allowing a more direct investigation of the translational value of animal addiction models.

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Disclosure/Conflict of Interest

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Authors Contribution

LMH wrote the first draft of the manuscript, obtained the data and conducted the statistical analyses. MS obtained the data, analyzed the ^{1}H-MRS data, assisted with the interpretation of the findings, and revised the first draft of the manuscript. MV and KHP were involved in data collection and revised the first draft of the manuscript. MRB conducted the hair analyses and revised the first draft of the manuscript. MH and ES revised and edited the first draft of the manuscript and assisted with data interpretation. AH performed the ^{1}H-MRS data analysis, assisted with interpretation of the findings, and revised the first draft of the manuscript. BBQ designed the study, assisted with the interpretation of the findings, and revised and edited the first draft and the revision of the manuscript. All authors critically reviewed content and approved final version for publication.

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Titles and legends to figures

Figure 1. Prototypical projections of 2D J-PRESS ^{1}H magnetic resonance spectroscopy spectra acquired in the bilateral pregenual anterior cingulate cortex (A) and the right dorsolateral prefrontal cortex (B) of a healthy control subject. The red lines represent the model fit, the blue lines the data, and the green lines the residual fit. mI, myo-inositol; Cho, choline; Cr, creatine; Gln, glutamine; Glu, glutamate; NAA, *N*-acetylaspartate.

Figure 2. A simulated in vivo 2D JPRESS spectrum from the pregenual anterior cingulate cortex voxel, the spectral fit, and the fit residue are presented.

Figure 3. (A) Scatterplots of glucose/total creatine ratios measured in the bilateral pregenual anterior cingulate cortex of 10 healthy controls and 16 cocaine users. The mean (indicated by the bar) ± SD was 5939.52±1885.42 and 7311.20±2240.28, respectively. (B) Scatterplots of total choline/total creatine ratios measured in the right dorsolateral prefrontal cortex of 18 healthy controls and 13 cocaine users. The mean (indicated by the bar) ± SD was 3832.61±406.02 and 4510.87±863.93, respectively.

Figure 4. (A) Scatterplots of glutamate/total creatine ratios measured in the bilateral pregenual anterior cingulate cortex of 17 healthy controls and 18 cocaine users. The mean (indicated by the bar) ± SD was 32300.81 ±6672.97 and 33045.94 ±10161.92, respectively. (B) Scatterplots of glutamate/total creatine ratios measured in the right dorsolateral prefrontal cortex of 18 healthy controls and 13 cocaine users. The mean ± SD was 36746.11 ±8876.64 and 40117.84 ±9783.55, respectively. (C) Scatterplots of glutamine/total creatine ratios measured in the bilateral pregenual anterior cingulate cortex of 17 healthy controls and 17 cocaine users. The mean (indicated by the bar) ± SD was 6494.12 ±1853.79 and 6761.57 ±1504.29, respectively. (D) Scatterplots of glutamine/total

creatine ratios measured in the right dorsolateral prefrontal cortex of 17 healthy controls and 13 cocaine users. The mean ± SD was 5920.72 ±2012.34 and 7048.24 ±2472.53, respectively.

Figure 5. Correlations between drug use variables and metabolite/total creatine ratios. Higher weekly cocaine use in grams and higher cocaine concentrations in the hair samples (pg/mg) were associated with lower glutamine/total creatine ratios in the pregenual anterior cingulate cortex (A and B) and lower myo-inositol/total creatine ratios in the dorsolateral prefrontal cortex of cocaine users (C and D).

Table 1. Demographic data and legal drug consumption (means and standard deviations, number of subjects and percent)

	Controls (n=18)	**Cocaine users (n=18)**	**Value[a]**	***p*[a]**	***df***
Age	35.78 (±8.32)	36.17 (±7.64)	-0.15	0.89	34
Body Mass Index (kg/m^2)	23.81 (±2.72)	25.09 (±3.51)	-1.22	0.23	34
Years of education	10.67 (±1.97)	10.50 (±1.89)	0.26	0.80	34
Beck Depression Inventory	5.28 (±6.48)	5.89 (±3.91)	-0.34	0.73	34
SCL-90-R Global Severity Index	0.29 (±0.41)	0.38 (±0.24)	-0.77	0.45	34
Cocaine Craving Questionnaire (sum)	-	20.06 (±6.03)	-	-	-
Smoking Status (yes/no)	12, 6 (67, 33%)	13, 5 (72, 28%)	0.13[b]	0.72[b]	1
FTND (sum)	2.83 (±2.79)	4.85 (±3.21)	-1.67	0.11	23
Alcohol use					
Grams per week	109.37 (±108.95)	262.00 (±251.48)	-2.36	**0.03**	34
Years of use	17.22 (±8.61)	17.58 (±6.46)	-0.14	0.89	34
Age of alcohol onset	18.56 (±5.75)	18.58 (±4.03)	-0.02	0.99	34
Last consumption (days)	7.67 (±13.69)	3.54 (±4.57)	1.22	0.24	34
Tobacco use					
Cigarettes per week	58.92 (±64.45)	85.19 (±73.74)	-1.11	0.28	34
Years of use	11.53 (±10.80)	12.06 (±9.18)	0.26	0.80	34
Age of smoking onset	24.25 (±10.72)	25.50 (±12.05)	-0.33	0.74	34
Last consumption (hours)	13.33 (±21.68) n=12	123.86 (±320.35) n=13	-1.24	0.24	23

[a]Independent T-test, [b]Chi^2-test for frequency data. SCL-90-R, Symptom Check List 90-R; FTND, Fagerström Test for Nicotine Dependence.

Table 2. Illegal drug use patterns (means and standard deviations)

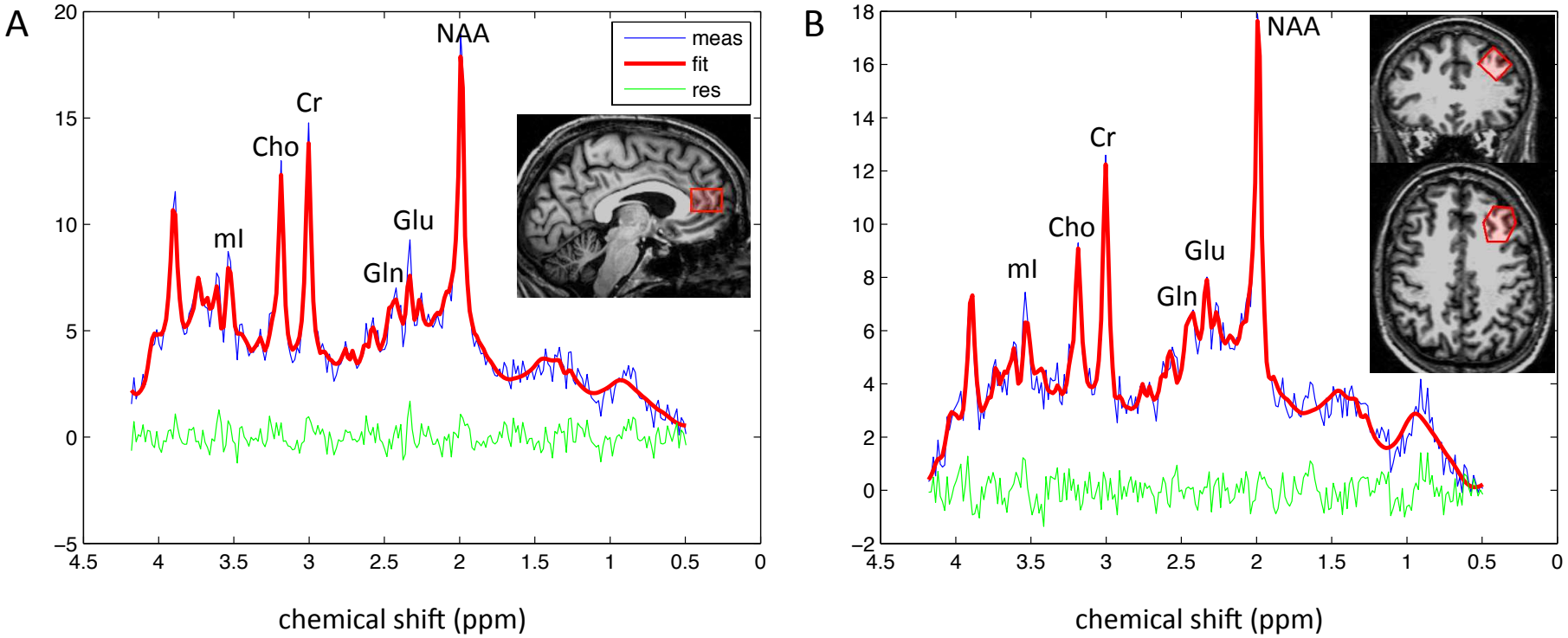
	Controls (n=18)	**Cocaine users (n=18)**
Cocaine		
Times per week	0.00 (±0.00)	1.28 (±1.39)
Grams per week	0.00 (±0.00)	1.46 (±1.36)
Years of use	0.00 (±0.00)	10.14 (±5.55)
Age of cocaine use onset	-	26.03 (±6.32)
Cumulative dose (grams)	0.00 (±0.00)	1056.56 (±977.50)
Last consumption (days)	-	7.60 (±6.84)
Total cocaine in hair (pg/mg)	0.00 (±0.00)	22770 (±23560)
Ethylcocaine in hair (pg/mg)	0.00 (±0.00)	1490 (±2710)
Urine toxicology (pos./neg.)	0, 100 (0, 100%)	8, 10 (44, 56%)
Cannabis		
Grams per week	0.15 (±0.42)	1.07 (±1.92)
Years of use	4.69 (±7.37)	7.83 (±8.67)
Age of cannabis onset	31.08 (±9.82)	28.33 (±11.25)
Cumulative dose (grams)	171.30 (±283.25)	1563.85 (±2689.90)
Last consumption (days)	50.76 (±74.39) n=5	3.81 (±2.14) n=8
Urine toxicology (pos./neg.)	2, 16 (13, 89%)	6, 12 (33, 67%)
Amphetamine		
Grams per week	0.00 (±0.00)	0.0043 (±0.01)
Years of use	0.00 (±0.00)	0.64 (±1.30)
Cumulative dose (grams)	0.00 (±0.00)	10.77 (±25.86)
Last consumption (days)	-	76.00 (±40.22) n=3
Amphetamine in hair (pg/mg)	0.00 (±0.00)	12 (±30) n=3
Methamphetamine in hair (pg/mg)	0.00 (±0.00)	0.00 (±0.00)
MDMA		
Pills per week	0.00 (±0.00)	0.03 (±0.06)
Years of use	0.00 (±0.00)	3.58 (±4.94)
Cumulative dose (pills)	0.00 (±0.00)	24.88 (±63.74)
Last consumption (days)	-	70.63 (±47.67) n=7
MDMA in hair (pg/mg)	0.00 (±0.00)	1644 (±4859) n=6
MDA in hair (pg/mg)	0.00 (±0.00)	68 (±259) n=2

Consumption per day/week captures the past six months, duration of use and cumulative dose are averaged within the total group. Last consumption is averaged only for subjects who used the drug in the past six months. In this case, sample size is shown. The hair analysis was performed on two hair samples (each 3 cm in length) per participant capturing drug use over the past six months. Concentrations were averaged over the two samples. If the hair sample was not long enough, only one sample was analyzed (3 cm, 3 months). MDMA, 3,4-methylenedioxy-N-methylamphetamine; MDA, 3,4-methylenedioxyamphetamine.

Table 3. Tissue composition and ^{1}H-MRS metabolite/total creatine ratios (means and standard errors)

	Pregenual Anterior Cingulate Cortex					Dorsolateral Prefrontal Cortex				
	Controls (n=17)	**Cocaine users (n=18)**	**T/F value**	**p value**	**df/df$_{err}$**	**Controls (n=18)**	**Cocaine users (n=13)**	**T/F value**	**p value**	**df/df$_{err}$**
Tissue composition %[a]										
Gray matter	69.57 (±0.90)	68.63 (±1.18)	0.63	0.54	33	53.92 (±1.13)	51.11 (±1.49)	1.54	0.14	29
White matter	11.89 (±0.50)	11.43 (±0.74)	0.51	0.61	33	36.03 (±2.13)	40.02 (±2.33)	-1.25	0.22	29
Cerebrospinal fluid	18.35 (±0.81)	19.68 (±1.13)	-0.94	0.35	33	7.54 (±0.89)	7.29 (±1.56)	0.15	0.88	29
Metabolites[b]										
Total creatine[c]	7.33 (±0.15)	7.16 (±0.15)	0.66	0.43	1/29	6.43 (±0.15)	6.39 (±0.18)	0.04	0.85	1/25
Glutamate	32703.44 (±2094.89)	32665.67 (±2034.50)	0.00	0.50	1/29	37588.31 (±2342.15)	38951.72 (±2790.62)	0.13	0.36	1/25
Glutamine	6483.22 (±400.69)	6772.47 (±400.69) n=17	0.25	0.31	1/28	6196.01 (±555.94) n=17	6688.24 (±642.72)	0.31	0.29	1/24
N-acetylaspartate	27275.94 (±1053.25)	27292.15 (±1022.89)	0.00	0.50	1/29	33096.86 (±1230.14)	34973.83 (±1465.69)	0.90	0.18	1/25
Total choline	4748.84 (±129.74)	5057.53 (±126.00)	2.85	**0.05**	1/29	3902.68 (±149.55)	4413.85 (±178.18)	4.49	**0.02**	1/25
γ-aminobutyric acid	3748.31 (±551.07) n=10	3856.42 (±584.03) n=9	0.02	0.45	1/13	5924.69 (±1039.13) n=13	4125.94 (±1286.96) n=9	1.02	0.17	1/16
Myo-inositol	18058.59 (±636.51)	18509.53 (±618.16)	0.25	0.31	1/29	16408.44 (±1439.02)	20170.85 (±1714.57)	2.63	0.06	1/25

[a]Unpaired T-test, [b]ANCOVA with age, smoking status, gray matter volume, and white matter volume as covariates (one-tailed tests), [c]referenced to H_2O.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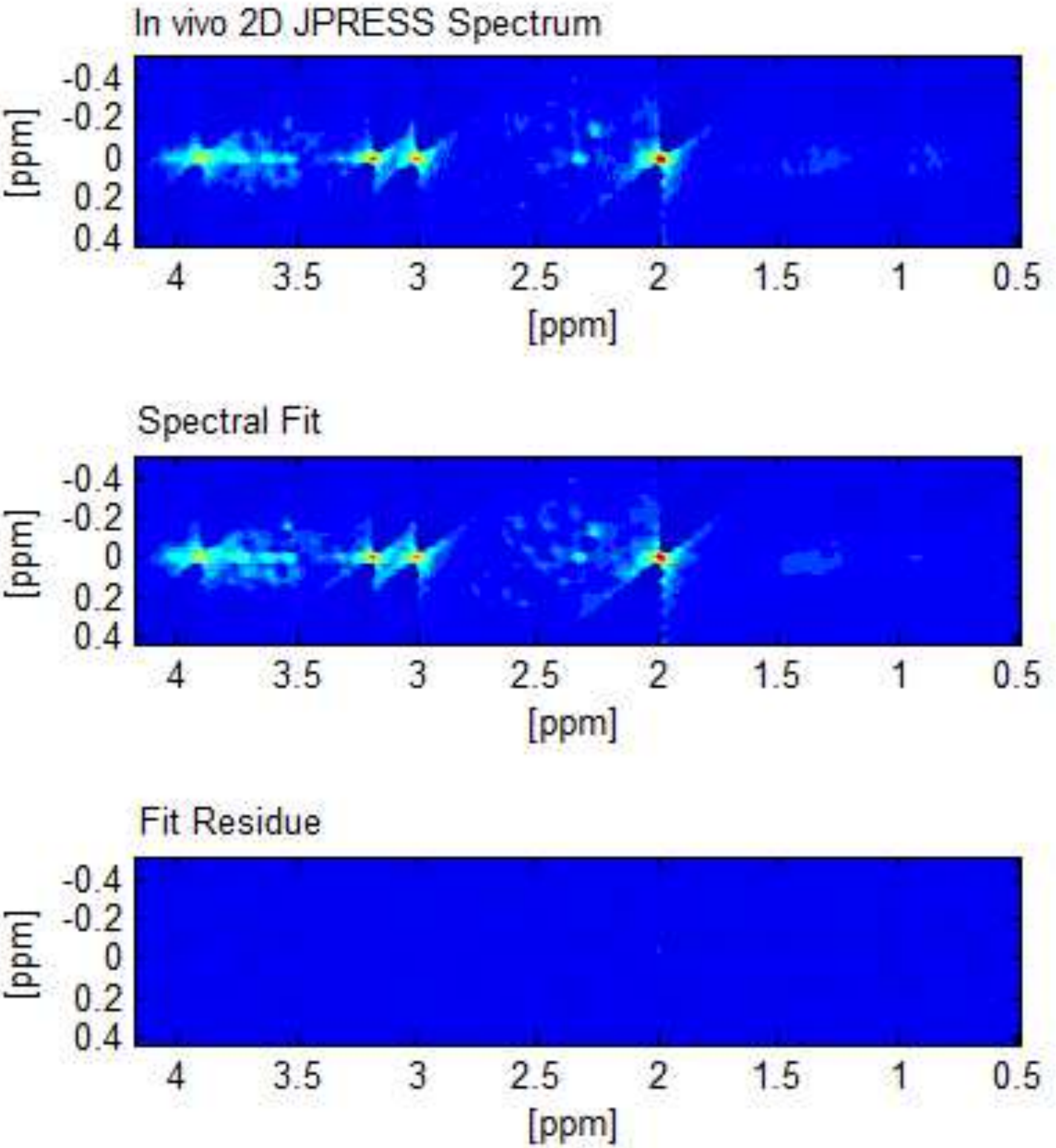
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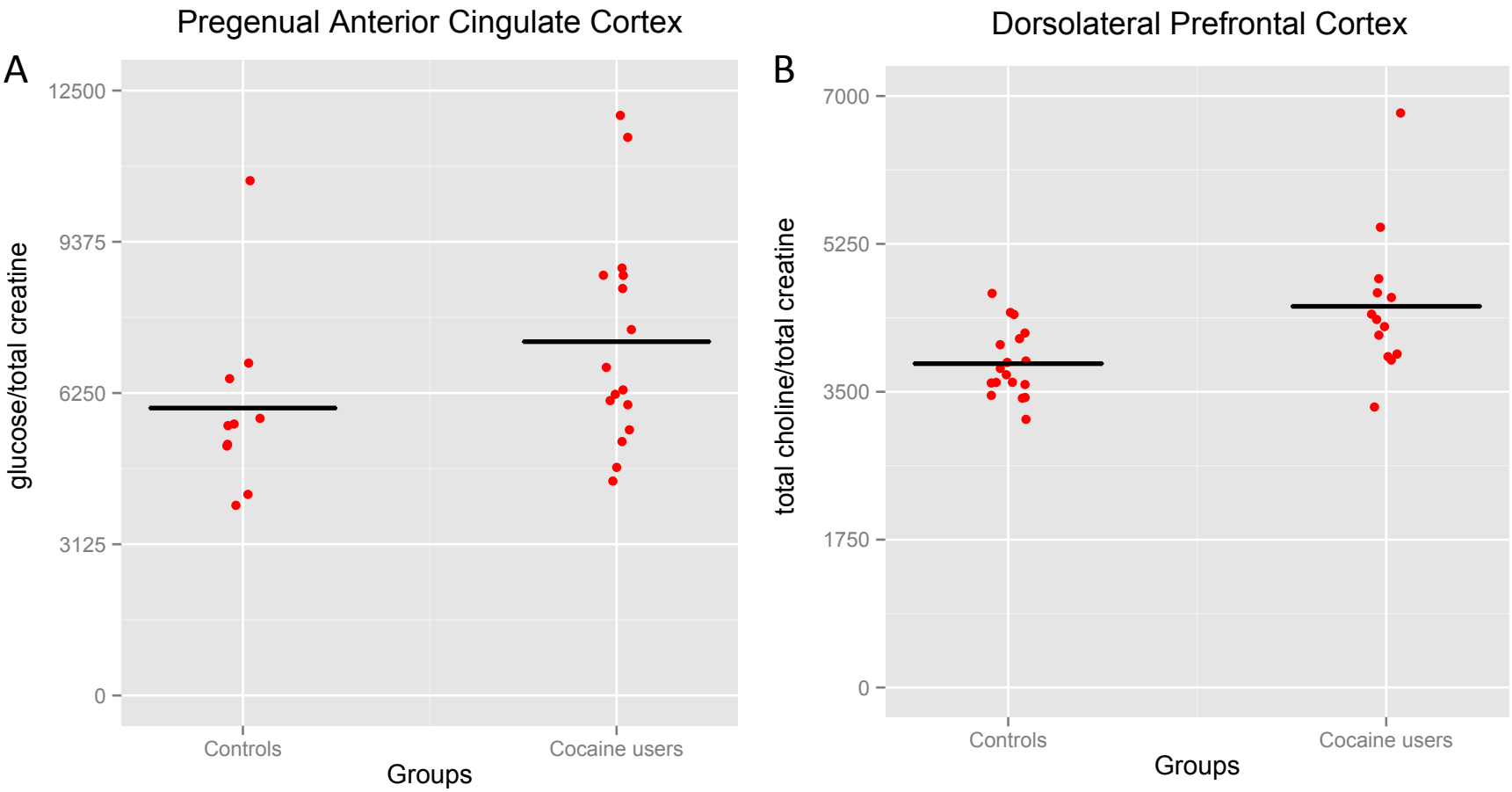
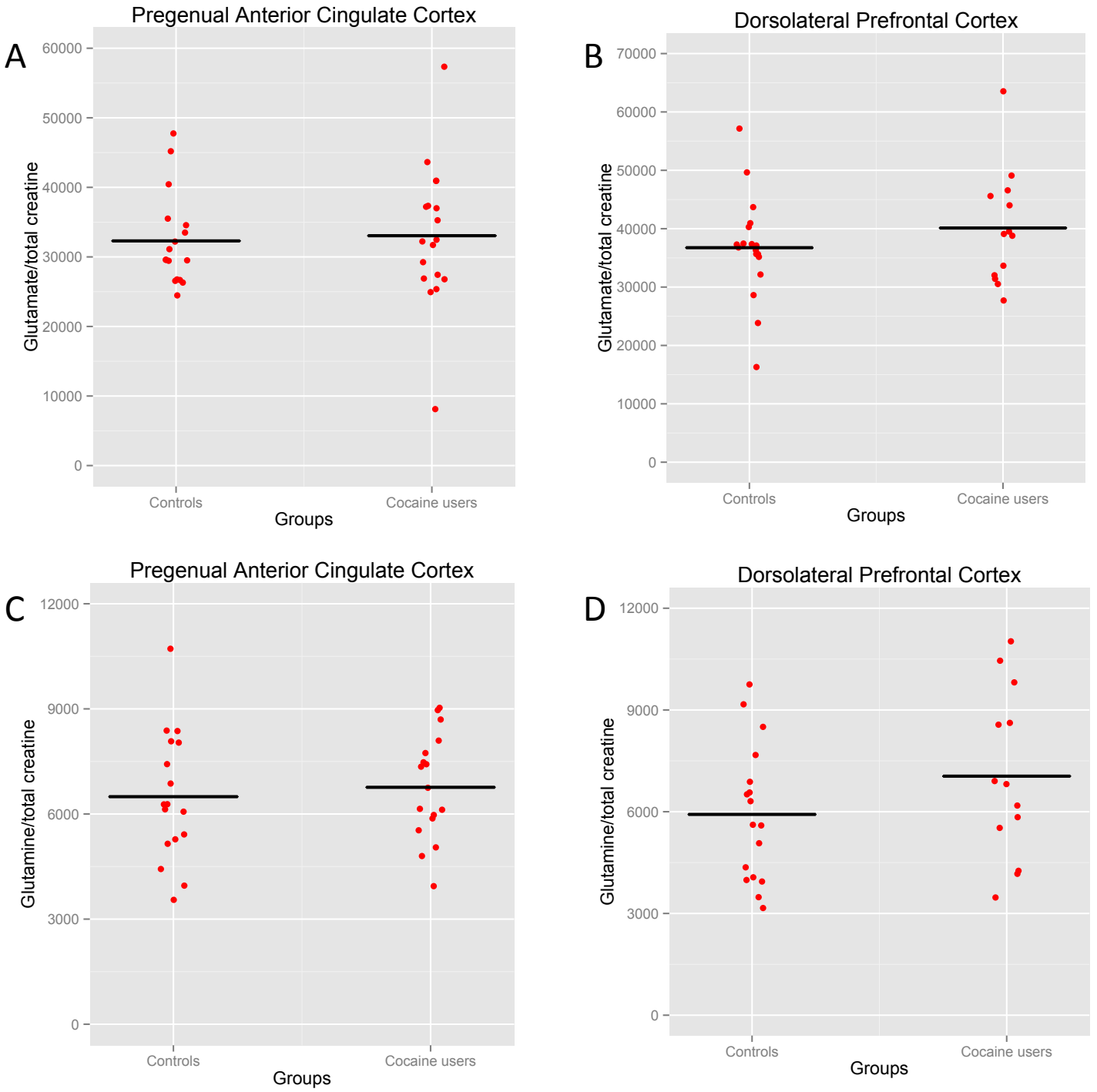
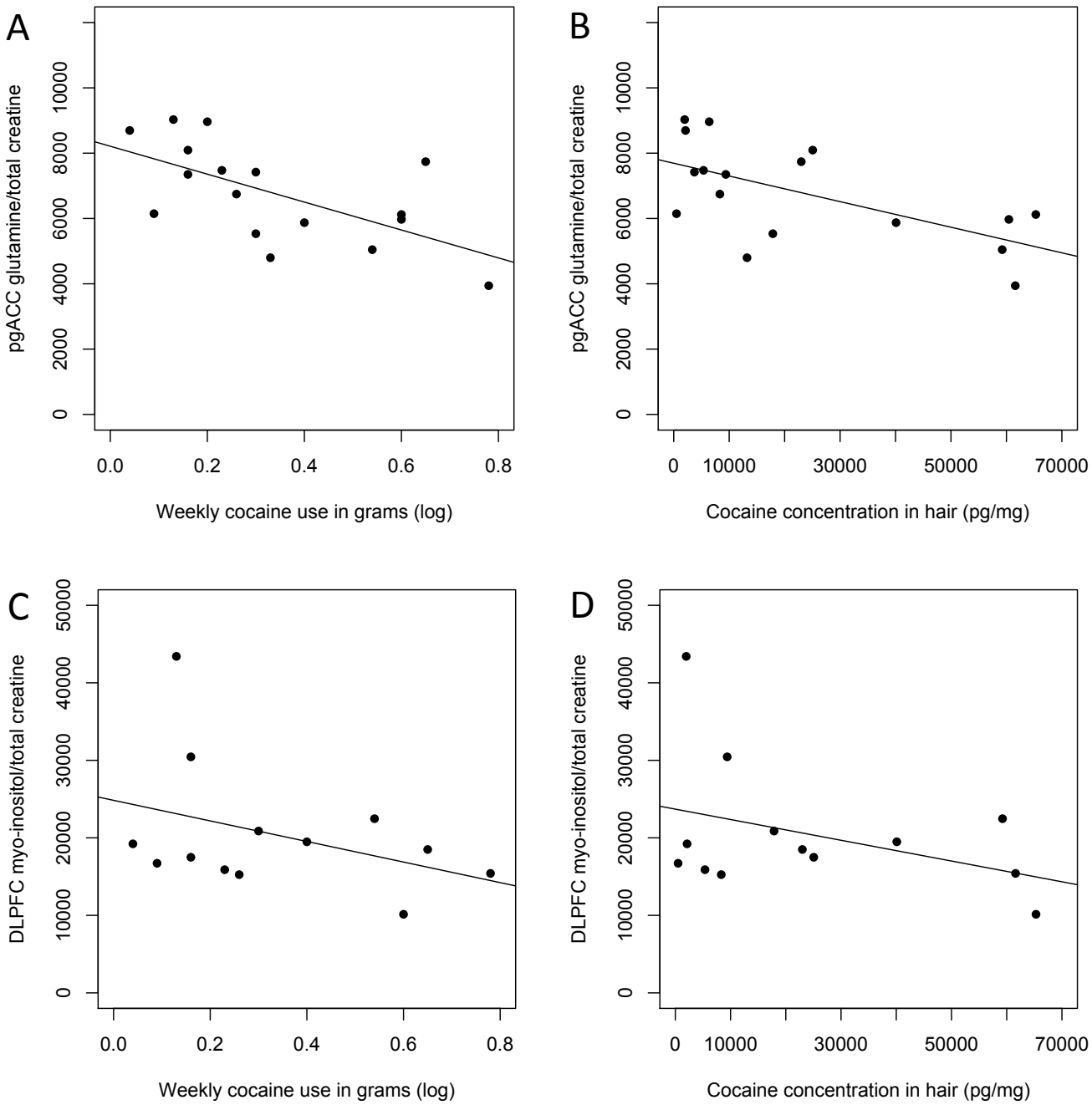

A
Pregenual Anterior Cingulate Cortex
glucose/total creatine
12500
9375
6250
3125
0
Controls
Cocaine users
Groups
B
Dorsolateral Prefrontal Cortex
total choline/total creatine
7000
5250
3500
1750
0
Controls
Cocaine users
Groups


A
Pregenual Anterior Cingulate Cortex
Glutamate/total creatine
60000
50000
40000
30000
20000
10000
0
Controls
Cocaine users
Groups
B
Dorsolateral Prefrontal Cortex
Glutamate/total creatine
70000
60000
50000
40000
30000
20000
10000
0
Controls
Cocaine users
Groups
C
Pregenual Anterior Cingulate Cortex
Glutamine/total creatine
12000
9000
6000
3000
0
Controls
Cocaine users
Groups
D
Dorsolateral Prefrontal Cortex
Glutamine/total creatine
12000
9000
6000
3000
0
Controls
Cocaine users
Groups


A
pgACC glutamine/total creatine
0
2000
4000
6000
8000
10000
0.0
0.2
0.4
0.6
0.8
Weekly cocaine use in grams (log)
B
pgACC glutamine/total creatine
0
2000
4000
6000
8000
10000
0
10000
30000
50000
70000
Cocaine concentration in hair (pg/mg)
C
DLPFC myo-inositol/total creatine
0
10000
20000
30000
40000
50000
0.0
0.2
0.4
0.6
0.8
Weekly cocaine use in grams (log)
D
DLPFC myo-inositol/total creatine
0
10000
20000
30000
40000
50000
0
10000
30000
50000
70000
Cocaine concentration in hair (pg/mg)

Supplemental Information

Glutamatergic and neurometabolic alterations in chronic cocaine users measured with ^{1}H magnetic resonance spectroscopy

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Materials and methods

Participants were recruited through advertisements in local newspapers, internet platforms, drug prevention and treatment centers, psychiatric hospitals, and word-of-mouth communication (for details see Hulka *et al.* 2014).

Reference

Hulka LM, Treyer V, Scheidegger M, Preller KH, Vonmoos M, Baumgartner MR, Johayem A, Ametamey SM, Buck A, Seifritz E, Quednow BB (2014) Smoking but not cocaine use is associated with lower cerebral metabotropic glutamate receptor 5 density in humans. Mol Psychiatry 19:625-632.

Table S1. ^{1}H-MRS exploratory metabolite ratios with total creatine as reference (means and standard errors)

	Pregenual Anterior Cingulate Cortex					Dorsolateral Prefrontal Cortex				
	Controls (n=17)	**Cocaine users (n=18)**	**F value**	**p value**	**df/df$_{err}$**	**Controls (n=18)**	**Cocaine users (n=13)**	**F value**	**p value**	**df/df$_{err}$**
NAAG	2248.87 (±281.35) n=16	2306.39 (±291.31) n=15	0.02	0.89	1/25	2457.92 (±280.11) n=16	2759.74 (±345.63) n=11	0.41	0.53	1/21
Glucose	5758.06 (±582.98) n=10	7424.61 (±456.85) n=16	4.89	**0.04**	1/20	6397.05 (±513.13) n=10	6252.20 (±544.73) n=9	0.03	0.86	1/13
Lactate	4249.38 (±453.24) n=16	4354.46 (±439.31) n=17	0.03	0.87	1/27	5421.22 (±900.57)	8372.97 (±1100.21) n=12	3.76	0.07	1/23
Scyllo-inositol	1887.50 (±151.20)	1749.34 (±146.84)	0.42	0.52	1/29	1655.29 (±174.36)	1854.44 (±207.75)	0.50	0.49	1/25
Taurine	6184.27 (±389.12) n=16	5313.87 (±377.23) n=17	2.52	0.12	1/27	5414.22 (±372.39) n=16	5997.27 (±435.55) n=12	0.95	0.34	1/22
Glycine	3373.96 (±329.02)	3067.60 (±329.02) n=17	0.43	0.52	1/28	3319.16 (±246.08) n=16	2988.60 (±318.04) n=10	0.62	0.44	1/20
Glutathione	7866.46 (±462.55)	7982.70 (±449.21)	0.03	0.86	1/29	7684.90 (±601.11)	8671.42 (±716.21)	1.04	0.32	1/25
Phosphoethanolamine	6044.10 (±833.35) n=14	7329.40 (±833.35) n=14	1.18	0.29	1/22	7210.72 (±788.07) n=15	7460.75 (±940.21) n=11	0.04	0.85	1/20
Aspartate	11572.12 (±868.96)	11440.43 (±843.90)	0.01	0.92	1/29	15616.44 (±1016.73)	15164.99 (±1211.41)	0.08	0.79	1/25
Ascorbic acid	8191.57 (±526.91)	8825.12 (±511.72)	0.73	0.40	1/29	6734.61 (±656.26)	8358.91 (±781.92)	2.36	0.14	1/25

ANCOVA with age, smoking status, grey matter volume, and white matter volume as covariates. NAAG, *N*-acetylaspartylglutamate.

Table S2. ^{1}H-MRS metabolite ratios with H_2O as reference (means and standard errors)

	Pregenual Anterior Cingulate Cortex					Dorsolateral Prefrontal Cortex				
	Controls (n=17)	**Cocaine users (n=18)**	**T/F value**	**p value**	**df/df$_{err}$**	**Controls (n=18)**	**Cocaine users (n=13)**	**T/F value**	**p value**	**df/df$_{err}$**
Glutamate	8.73 (±0.52)	8.46 (±0.50)	0.14	0.36	1/29	8.99 (±0.48)	8.81 (±0.58)	0.05	0.41	1/25
Glutamine	1.73 (±0.11)	1.77 (±0.11) n=17	0.09	0.39	1/28	1.49 (±0.13) n=17	1.53 (±0.16)	0.03	0.44	1/24
NAA	7.25 (±0.14)	7.07 (±0.14)	0.74	0.20	1/29	7.89 (±0.15)	7.90 (±0.18)	0.00	0.49	1/25
Total choline	1.27 (±0.04)	1.33 (±0.04)	1.26	0.14	1/29	0.93 (±0.02)	1.00 (±0.03)	3.74	**0.04**	1/25
GABA	0.99 (±0.17) n=10	1.09 (±0.18) n=9	0.16	0.35	1/13	1.36 (±0.21) n=13	0.94 (±0.27) n=9	1.31	0.14	1/16
myo-inositol	4.81 (±0.12)	4.83 (±0.12)	0.02	0.45	1/29	3.93 (±0.29)	4.52 (±0.35)	1.56	0.11	1/25
NAAG	0.57 (±0.06) n=16	0.06 (±0.06) n=15	0.09	0.77	1/25	0.58 (±0.06) n=16	0.62 (±0.08) n=11	0.16	0.70	1/21
Glucose	1.44 (±0.14) n=10	1.97 (±0.11) n=16	7.94	**0.01**	1/20	1.45 (±0.08) n=10	1.43 (±0.08) n=9	0.03	0.87	1/13
Lactate	1.12 (±0.12) n=16	1.14 (±0.12) n=17	0.01	0.93	1/27	1.29 (±0.21) n=17	1.95 (±0.25) n=12	3.69	0.07	1/23
Scyllo-inositol	0.50 (±0.04)	0.46 (±0.04)	0.61	0.44	1/29	0.39 (±0.04)	0.41 (±0.04)	0.07	0.79	1/25
Taurine	1.68 (±0.12) n=16	1.39 (±0.12) n=17	2.71	0.11	1/27	1.30 (±0.08) n=16	1.35 (±0.10) n=12	0.10	0.75	1/22
Glycine	0.91 (±0.10)	0.84 (±0.10) n=17	0.27	0.61	1/28	0.80 (±0.06) n=16	0.70 (±0.08) n=10	0.88	0.36	1/20
Glutathione	2.12 (±0.15)	2.11 (±0.14) n=18	0.00	0.95	1/29	1.84 (±0.12)	1.94 (±0.15)	0.30	0.59	1/25
Phosphoethanolamine	1.59 (±0.24) n=14	1.99 (±0.24) n=14	1.40	0.25	1/22	1.68 (±0.17) n=15	1.66 (±0.20) n=11	0.00	0.95	1/20
Aspartate	3.10 (±0.22)	2.99 (±0.22)	0.11	0.75	1/29	3.76 (±0.22)	3.40 (±0.27)	0.97	0.33	1/25
Ascorbid acid	2.18 (±0.15)	2.32 (±0.14)	0.43	0.52	1/29	1.62 (±0.13)	1.88 (±0.16)	1.41	0.25	1/25

ANCOVA with age, smoking status, grey matter volume, and white matter volume as covariates. NAA, *N*-acetylaspartate; GABA, γ-aminobutyric acid; mI, myo-inositol; NAAG, *N*-acetylaspartylglutamate.